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A Simple and Effective Isocratic HPLC Method for Fast Identification and Quantification of Surfactin

(Kaedah Isokratik HPLC Ringkas dan Berkesan bagi Pengenalpastian dan Kuantifikasi Surfaktin dengan Cepat)

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ABSTRACT

The aim of this study was to establish a simple, accurate and reproducible method for the identification and quantification of surfactin using high-performance liquid chromatography (HPLC). Previously reported method of identification and quantification of surfactin were time consuming and requires a large quantity of mobile phase. The new method was achieved by application of Chromolith® high performance RP-18 (100 × 4.6 mm, 5 µm) as the stationary phase and optimization of mobile phase ratio and flow rate. Mobile phase consisted of acetonitrile (ACN) and 3.8 mM trifluroacetic acid (TFA) solution of 80:20 ratio at flow rate of 2.2 mL/min was obtained as the optimal conditions. Total elution time of the obtained surfactin peaks was four times quicker than various methods previously reported in the literature. The method described here allowed for fine separation of surfactin in standard sample (98% purity) and surfactin in fermentation broth.

Keywords: High-performance liquid chromatography (HPLC); isocratic; mobile phase; stationary phase; surfactin

ABSTRAK

Tujuan kajian ini adalah untuk menyediakan satu kaedah yang mudah, tepat dan boleh diulang untuk mengenal pasti dan mengkuantifikasi surfaktin menggunakan kromatografi cecair berprestasi tinggi (HPLC). Sebelum ini dilaporkan kaedah kenal pasti dan kuantifikasi surfaktin memerlukan masa yang panjang dan amaun fasa bergerak yang banyak. Kaedah baru telah diperoleh dengan menggunakan kolum Chromolith® high performance RP-18 (100 × 4.6 mm, 5 µm) sebagai fasa pegun dan pengoptimuman nisbah dan kadar aliran fasa bergerak. Fasa bergerak terdiri daripada cecair asetonitril (ACN) dan 3.8 mM asid trifluroacetic (TFA) dengan nisbah 80:20 pada kadar aliran 2.2 mL/min sebagai kadar optimum. Masa analisis yang diperoleh adalah empat kali lebih pantas daripada kaedah yang dilaporkan sebelum ini. Kaedah yang diguna pakai di sini dapat memisahkan piawai surfaktin (98% ketulenan) dan surfaktin dalam sampel fermentasi dengan baik.

Kata kunci: Fasa bergerak; fasa pegun; isokratik; kromatografi cecair prestasi tinggi (HPLC); surfaktin

INTRODUCTION

Surfactants, which can be either chemically or biologically produced, are defined as surface active agents that have wide ranging and attractive properties (Al-Araji et al. 2007). Surfactants produce by biological synthesis are known as biosurfactants and can be generated by a variety of bacteria, yeasts and fungi through utilization of various carbon feedstocks such as sugars and oil (Chen et al. 2007). Biosurfactants are usually produced during the stationary phase and excreted as secondary metabolites during growth in microbial culture broth (Georgiou et al. 1992; Wei et al. 2007).

Biosurfactants offer various advantages over chemical surfactants, including being less toxic, but more biodegradable and environmentally friendly, as well as able to maintain their physico-chemical properties at different temperatures and pH (Mulligan 2005). Due to these attractive properties, there has been increased interest in biosurfactants for applications in various fields, such as food, medical, pharmaceutical, cosmetics and agriculture industries (Banat et al. 2000). In addition, biosurfactants possess several unique therapeutic properties of biomedical importance (Singh & Cameotra 2004) and can exert anti-adhesive activity against several pathogenic microorganisms (Heinemann et al. 2000). These features make biosurfactants favourable alternatives to chemically synthesised surfactants for a variety of applications.

Surfactin also exhibit many pharmacological activities, such as antimicrobial, antiviral, anti-inflammatory and antimycoplasma activity, as well as the ability to inhibit fibrin clot formation and haemolysis. Additionally, they have antitumor activity against Ehrlich's ascites carcinoma cells and can inhibit the cyclic adenosine 3, 5-monophosphate phosphodiesterase (Davies et al. 2001; Fernandes et al. 2007). Research on the formulation, characterization and pharmacokinetics of surfactin

showed an accurate, effective, quick and reproducible analytical method for the identification and quantification of surfactin. Identification and quantification of surfactin produced by *Bacillus subtilis* ATCC 21332 is complex since it produces a series of isoforms that differ slightly in their physiochemical properties due to variations in the chain length and branching of its hydroxy fatty acid component (Hosono & Suzuki 1983) as well as substitutions of the amino acid components of the peptide ring (Peypoux 1991).

High-performance liquid chromatography (HPLC) is one of the best methods used to identify and quantify surfactin and has been described in various reports in the literature. Most of the previous methods used employ gradient elution, which causes base line shifting and is time consuming, with a total sampling time of more than 30 min (Fonseca et al. 2007; Isa et al. 2007; Lin et al. 1998; Wei & Chi 2002). A delay in total sampling time will result in the use of increasing amounts of the mobile phase, which is not necessarily cost effective.

Earlier studies on surfactin identification and quantification involved measuring the surface and interfacial tension of fermentation culture broth or by thin-layer chromatography (Cooper et al. 1981). However, neither of this method is satisfactory for quantitative analysis of surfactin. In contrast, the HPLC assay described in this paper is specific for surfactin identification and quantification and is highly sensitive, quick and reproducible. There is a demand for a quicker and more reliable HPLC method for qualitative and quantitative measurements of surfactin with variable formulation. The objective of the research presented in this paper was to develop an improved HPLC method, which offers a well separated individual peak with the consistent base line that is reproducible, accurate and has a minimal duration of total elution time. In this study, a novel isocratic HPLC method with variable wavelength detector (VWD) was developed and is considered an improved method for qualitative and quantitative analysis of surfactin in comparison to various methods previously reported.

Methods

Apparatus

An Agilent 1200 series HPLC system consisting of a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and variable wavelength detector was used. Data acquisition was performed using Chemstation software (Agilent Corporation).

Surfactin Standard and Chemicals

Surfactin standard of 98% purity was purchased from Sigma-Aldrich (Sigma, USA). Acetonitrile (ACN) and Trifluroacetic acid (TFA) solution of HPLC grade were purchased from RCI Labscan (Thailand) and Merck (Germany), respectively. Deionised water was obtained from the Microbiology Laboratory of Universiti Sains Islam Malaysia (Negeri Sembilan, Malaysia). Deionised water was prepared using a system equipped with 0.2 μm filter.

Chromatographic Conditions

HPLC system equipped with a Chromolith® high performance RP-18 (100 × 4.6 mm, 5 μm) column was operated and maintained at 25°C. A mobile phase mixture consisting of an ACN and 3.8 mM TFA solution (ratio of 80:20) were pumped in an isocratic mode with a flow rate of 2.2 mL/min. The injection volume of surfactin was set at 30 μL and was detected through a VWD detector at 205 nm. Each analysis was completed within 8 min.

Preparation of Surfactin Stock and Standard Solutions

Methanolic surfactin stock standard solution was prepared at 5000 mg/L. Later, a series of surfactin solution of 10 to 1000 mg/L were prepared by dilution of the stock solution with methanol and were then stored at 4°C prior to use.

Preparation of the Mobile Phase Solution

A 3.8 mM TFA solution was prepared in a 1 L volumetric flask filled with deionised water and stirred until complete dissolution. Later, the TFA solution and ACN were filtered through 0.22 μm nylon filters and degassed prior to use.

Surfactin Calibration Curve

The obtained chromatograms were analysed using Chemstation software. The surfactin calibration curve was constructed by plotting graphs of the total peak area (TPA) against various concentrations of surfactin standard.

Sample Preparation

The commercial strain of *B. subtilis* ATCC 21332 used in this study was provided by the Microbiology Laboratory, Faculty of Science and Technology, University Sains Islam Malaysia. Bacterial strains were maintained on nutrient agar (NA) at 4°C prior to use. Two loopfuls of colonies were inoculated in 100 mL of Cooper's medium (4% (w/v) glucose and mineral salts) (Cooper et al. 1981), prepared in a 200 mL Erlenmeyer flasks and were incubated in an incubator shaker at 200 rpm at 30°C for 24 h. Then, 0.5 mL of the seed culture was inoculated into 200 mL of Cooper's media in a 500 mL Erlenmeyer flasks under the same condition (Wei & Chi 2002) for 96 h. Samples were withdrawn aseptically for further HPLC analysis.

Results and Discussion

Determination of Suitable Surfactin Separation Condition

The total elution time and separation of individual surfactin peaks was highly dependent upon the ratio of ACN and

3.8 mM TFA used in the mobile phase. Mixtures of trace amounts of TFA in deionised and ACN are suitable as a mobile phase for surfactin analysis and agrees with similar work on quantitative measurements of surfactin that has been previously reported (Fonseca et al. 2007; Isa et al. 2007; Wei & Chi 2002). Acceptable resolution of surfactin peaks was not obtained with ACN and TFA solution ratios of about 60:40, 70:30 or 75:25. The optimum separation of surfactin individual peak was achieved with mobile phase consisting 80:20 ratio of ACN and 3.8 mM TFA, respectively. This ratio was also similar to multiple previously reported findings (Fonseca et al. 2007; Hsieh et al. 2004; Isa et al. 2007; Joshi et al. 2008). The presence of TFA in deionised water as mobile phase could suppress the dissociation the structure of surfactin isoforms. The retention times for surfactin were recorded at 2.13, 2.57, 2.78, 3.43, 3.70, 4.14, 4.65, 5.06, 6.27, 6.46 and 7.18 min, as shown in Figure 1. The HPLC was run for 8 min, which resulted in both satisfactory resolution of surfactin peaks and quicker total elution time compared to previously reported method (Fonseca et al. 2007; Isa et al. 2007; Lin et al. 1998; Wei & Chi 2002).

VALIDATION OF METHOD

Linearity The calibration curve graph for surfactin, shown in Figure 2, was constructed by plotting the total peak area (TPA) against various concentrations of surfactin standard. Satisfactory linearity was obtained in the range of 10 to 1000 mg/L ($y = 10.413\times$, $r^2 = 0.9935$, y = TPA, × is the amount of surfactin standard in mg/L). The equation derived from the graph represents the relationship of TPA to various concentrations of surfactin. Independent t-tests were performed to analyse various concentrations of surfactin standard. A significant difference was achieved between the results obtained by HPLC from increasing concentrations at a 95% confidence level ($p \leq 0.05$). The calibration curve exhibited good linearity over increasing concentrations of surfactin tested, with correlation coefficients (r^2) of 0.9935. The limit of detection (LOD) achieved for the surfactin standards was 5 mg/L per injection volume of 30 µL. The high precision and low LOD indicated that the proposed method was reliable for quantitative analysis of surfactin.

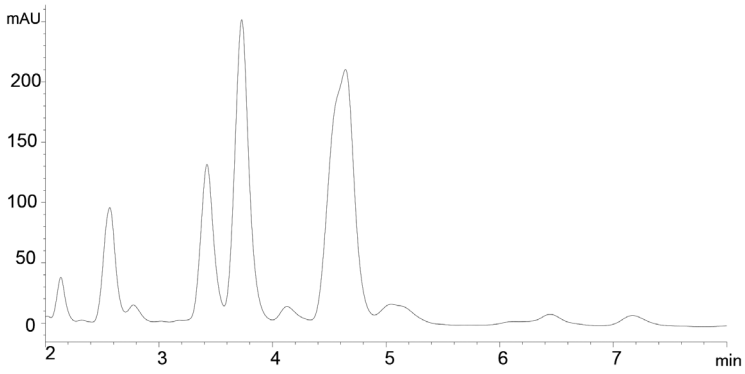

FIGURE 1. HPLC Chromatogram for surfactin standard concentration at 800 (mg/L)

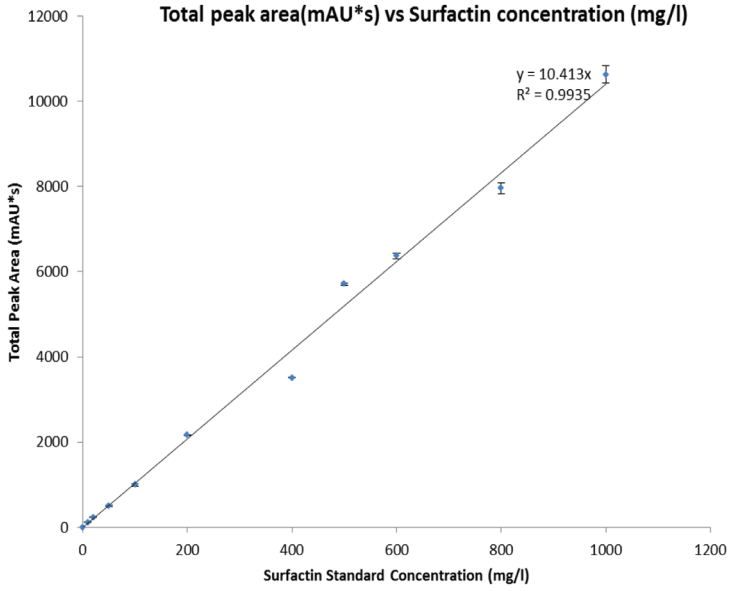

FIGURE 2. Plot of TPA versus surfactin standard

PRECISION

Precision of the proposed method was assessed by repeated injection of a surfactin standard solution of various concentrations. The relative standard deviation (RSD) used to assess instrumental precision and intra-assay precision shown in Table 1. The instrumental precision was measured by repeated injection of the same homogeneous sample while Intra-assay precision was determined by measuring independent surfactin standard solutions of varying concentration (Li et al. 2012). This was repeated for all of the standards. A correlation between the analyte peak area and surfactin standard at various concentrations was observed with $r^2 = 0.9933$ and $r^2 = 0.9929$ for standard curves for instrumental precision and intra-assay precision data as shown in Table 1, respectively, with $r^2 \approx 1$ for both. Meanwhile, the RSD value of both instrumental and intra-assay precision of the surfactin solution were below 2.5%, indicating both HPLC and the proposed method were highly precise, accurate and reproducible for quantitative analysis of surfactin (Li et al. 2012).

INTERMEDIATE PRECISION

Intermediate precision of the proposed method was determined by assessing intra-day and inter-day reproducibility. The results of intra- and inter-day reproducibility are listed in Table 2. The RSD values for all tested groups were approximately or less than 3%, which can be considered an acceptable value for quantitative analysis of surfactin (Li et al. 2012).

SENSITIVITY

The sensitivity of this HPLC method was determined by LOD and the limit of quantification (LOQ). LOD is the lowest analyte concentration detectable by HPLC for the proposed method. On the other hand, the LOQ is the lowest concentration that can be quantified accurately by the proposed method. The LOD and LOQ were 5 and 7 mg/L, respectively. Compared with other reported HPLC methods, the sensitivity of the described method was considerably improved.

TABLE 1. Instrumental precision and intra-assay precision (means ± SD)

Concentration (mg/L)	Instrumental precision[a]		Intra-assay precision[b]	
	Peak area (mAU*s)	RSD (%)	Peak area (mAU*s)	RSD (%)
0	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00
10	109.01 ± 2.03	1.86	102.37 ± 2.43	2.37
20	231.49 ± 2.40	1.04	228.47 ± 2.74	1.20
50	510.62 ± 1.88	0.37	503.34 ± 2.17	0.43
100	1015.89 ± 8.71	0.86	997.08 ± 2.59	0.26
200	2158.98 ± 5.57	0.26	2082.14 ± 10.53	0.51
400	3512.27 ± 13.49	0.38	3506.61 ± 21.86	0.61
500	5695.47 ± 3.53	0.06	5604.08 ± 12.63	0.22
600	6340.06 ± 22.83	0.36	6291.99 ± 14.36	0.23
800	7858.49 ± 24.58	0.31	7683.14 ± 23.02	0.30
1000	10495.13 ± 14.01	0.13	10473.77 ± 20.54	0.20

[a] Three injection of each sample (n=3)
[b] Three injection of each sample (n=3)

TABLE 2. Intra-day and inter-day reproducibility of the assay (means ± SD, n = 3)

Concentration (mg/L)	Intra-day reproducibility of peak area		Inter-day reproducibility of peak area	
	Average (mAU*s)	RSD (%)	Average (mAU*s)	RSD (%)
0	0.00 ± 0.00	0.00	0.00 ± 0.00	0 .00
10	97.64± 2.52	2.58	96.22 ± 2.64	2.75
20	231.94 ± 2.65	1.14	234.58 ± 4.49	1.90
50	480.66 ± 10.91	2.27	482.30 ± 8.30	1.72
100	984.05 ± 11.08	1.13	947.71 ± 8.76	0.92
200	2137.3 ± 11.24	0.53	2195.96 ± 14.13	0.64
400	3415.75 + 15.60	0.46	3253.88 ± 40.81	1.25
500	5718.64 ± 31.06	0.54	5813.31 ± 13.76	0.24
600	6376.41 ± 53.60	0.84	6439.32 ± 43.54	0.68
800	7882.73 ± 131.80	1.67	8077.60 ± 37.13	0.46
1000	11035.46 ± 50.67	0.46	12066.19 ± 90. 86	0.75

RECOVERY

The recovery measures the closeness between the theoretically added amount and the experimental value. It was performed by spiking the surfactin standard solution with a known concentration. The recovery of the sample at different concentrations was above 94% with RSD values below 3%, as shown in Table 3. The results indicated that surfactin can be fully recovered using the column proposed in this study.

CHROMATOGRAPHY OF SURFACTIN IN CULTURE BROTH OF *B. SUBTILIS* ATCC 21332

The proposed method was also tested with surfactin synthesized from culture broth of *B. subtilis* ATCC 21332. This fermentation broth also contained other fermentation products and residues such as proteins, sugar, lipid compounds and different types of amino acids (Pursell et al. 2004). The complex nature of *B. subtilis* ATCC 21332 culture broth makes identification and quantification of surfactin difficult. Furthermore, surfactin has different isoforms, which vary in carbon chain length and the peptide sequence (Oka et al. 1993) and depend on the bacterial strain, nutritional supply and environmental conditions applied during fermentation (Oka et al. 1993). Figure 3 shows a chromatogram of surfactin from culture broth of *B. subtilis* ATCC 21332. It has overall similarity to the surfactin standard chromatogram shown in Figure 1 in terms of the elution time and separation of surfactin individual peak respectively, with a total elution time of approximately 8 min. Determination of the surfactin concentration in the culture broth of *B. subtilis* ATCC 21332 was conducted by summing the area under all of the surfactin peaks in the chromatrogram (Figure 3) and then calculated using the equation y = 10.413×, obtained from Figure 2. The surfactin concentration in the culture broth of *B. subtilis* ATCC 21332 was 168.28±5.49 mg/L, as determined by performing the injection in triplicate.

CONCLUSION

An efficient and sensitive HPLC procedure for qualitative and quantitative analysis of surfactin has been successfully developed with total elution time of 8 min. Linearity of analysis was achieved up to at least 1000 mg/L of surfactin with more than 95% recovery. Reducing the total elution time of surfactin analysis is important for reducing the amount of mobile phase used, thereby making the method more cost effective and environmentally friendly. Furthermore, the method proposed in this research work can assist in reducing the total cost involving upstream and downstream processing methods of surfactin, which

TABLE 3. Recoveries of surfactin (means ± SD, *n*=3)

Concentration spike (mg/L)	Concentration detected (mg/L)	Recovery (%)	RSD (%)
10	9.52 ± 0.16	95.23	1.67
20	20.47 ± 0.29	102.37	1.43
50	47.05 ± 1.13	94.10	2.39
100	96.35 ± 2.05	96.35	2.13
200	204.42 ± 4.96	102.21	2.42
400	400.63± 4.35	100.16	1.08
500	500.91 ± 2.14	100.18	0.43
600	598.88 ± 5.27	99.81	0.88
800	803.76 ± 21.19	100.47	2.64
1000	1005.33 ± 21.17	100.53	2.11

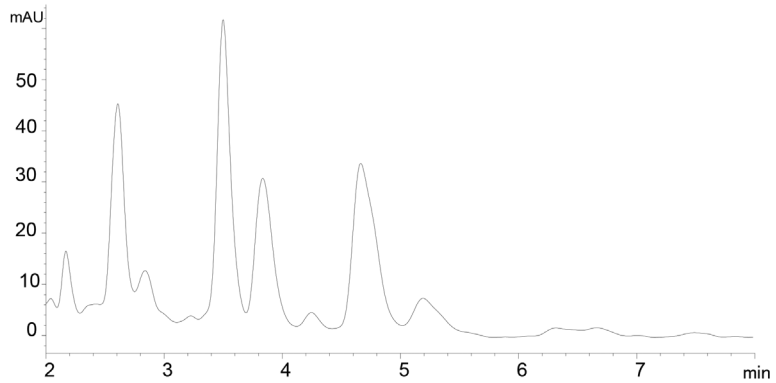

FIGURE 3. Chromatogram of surfactin in culture broth

can increase the competitiveness of the commercial uses of biosurfactants in relation to other chemical surfactants.

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REFERENCES

Al-Araji, L., Rahman, R.N.Z.R.A., Basri, M. & Salleh, A.B. 2007. Microbial surfactant. *Asia Pacific Journal of Molecular Biology and Biotechnology* 15: 99-105.

Banat, I.M., Makkar, R.S. & Cameotra, S.S. 2000. Potential commercial applications of microbial surfactants. *Applied Microbiology and Biotechnology* 53: 495-508.

Chen, C.Y., Bake, S.C. & Darton, R.C. 2007. The application of a high throughput analysis method for the screening of potential biosurfactant from natural sources. *Journal of Microbiological Methods* 70: 503-510.

Cooper, D.G., Macdonald, C.R., Duff, S.J.B. & Kosaric, N. 1981. Enhance production of surfactin from *Bacillus Subtilis* by continuous product removal and metal cation additions. *Journal of Applied and Environmental Microbiology* 42: 408-412.

Davies, D.A., Lynch, H.C. & Varley, J. 2001. The application of foaming for the recovery of surfactin from *B. subtilis* ATCC 21332 cultures. *Enzyme and Microbial Technology* 28: 346-354.

Fernandes, P.A.V., de Aruda, I.R., dos Santos, A.F.A.B., de Araujo, A.A., Maiour, A.M.S. & Ximenes, E.A. 2007. Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug bacteria. *Brazilian Journal of Microbiology* 38: 704-709.

Fonseca, R.R., Silva, A.J.R., De Franca, F.P., Cardoso, V.L. & Servulo, E.F.C. 2007. Optimzing carbon/nitrogen ratio for biosurfactant production by a *Bacillus subtilis* strain. *Applied Biochemistry and Biotechnology* 471: 137-140.

Georgiou, G., Lin, S.C. & Sharma, M.M. 1992. Surface-active compounds from microorganisms. *Biotechnology* 10: 60-65.

Heinemann, C., Hylckama, V., van Johan, E.T., Janssen, D.B., Busscher, H.J., van der Mei, H.C. & Reid, G. 2000. Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiology Letters* 190: 177-180.

Hosono, K. & Suzuki, H. 1983. Acylpeptides, the inhibitors of cyclic adenosine 3′,5′ monophosphate phosphodiesterase I. Purification, physicochemical properties and structures of fatty acid residues. *Journal of Antibiotics* 36: 667-673.

Hsieh, F.C., Li, M.C., Lin, R.C. & Kao, S.S. 2004. Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. *Current Microbiology* 49: 186-191.

Isa, M.H.M., Coraglia, D.E., Frazier, R.A. & Jauregi, P. 2007. Recovery and purification of from fermentation broth by a two-step ultrafiltration process. *Journal of Membrane Science* 296: 51-57.

Joshi, S., Bharucha, C. & Desai, A.J. 2008. Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B. *Bioresource Technology* 99: 4603-4608.

Li, D., Martini, N., Wu, Z. & Wen, J. 2012. Development of an isocratic HPLC method for catechin quantification and its application to formulation studies. *Fitoterapia* 83: 1267-1274.

Lin, S.C., Chen, Y.C. & Lin, Y.M. 1998. Genereal approach for the development of high-performance liquid chromatography methods for biosurfactant analysis and purification. *Journal of Chromatogram A*. 825: 149-159.

Mulligan, C.N. 2005. Environmental applications for biosurfactants. *Environment and Pollution* 133: 183-198.

Oka, K., Hirano, T., Homma, M., Ishii, H., Murakami, K., Mogami, S., Motizuki, A., Morita, H., Takeya, K. & Itokawa, H. 1993. Satisfactory separatin and MS-MS spectrometry of six surfactin isolated from *Bacillus subtilis natto*. *Chemical and Pharmaceutical Bulletin* 41: 1000-1002.

Peypoux, F., Bonmatin, J.M., Labb, H., Das, B.C., Ptak, M. & Michel, G. 1991. Isolation and characterization of a new variant of surfactin, the [Val7] surfactin. *European Journal of Biochemistry* 202: 101-106.

Pursell, M.R., Mendes-Tatsis, M.A. & Stuckey, D.C. 2004. Effect of fermentation broth and biosurfactants on mass transfer during liquid-liquid extraction. *Biotechnology and Bioengineering* 85: 155-165.

Singh, P. & Cameotra, S.S. 2004. Potential applications of microbial surfactants in biomedical sciences. *Trends in Biotechnology* 22: 142-146.

Wei, Y.H. & Chi, M. 2002. Mn^{2+} improves surfactin production by *Bacillus subtilis*. *Journal of Biotechnology Letters* 24: 479-482.

Wei, Y.H., Lai, C.C. & Chang, J.S. 2007. Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. *Process Biochemistry* 42: 40-45.

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